

A-to-G Hypermutation in the Genome of Lymphocytic Choriomeningitis Virus[†]

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The interferon-inducible adenosine deaminase that acts on double-stranded RNA (ADAR1-L) has been proposed to be one of the antiviral effector proteins within the complex innate immune response. Here, the potential role of ADAR1-L in the innate immune response to lymphocytic choriomeningitis virus (LCMV), a widely used virus model, was studied. Infection with LCMV clearly upregulated ADAR1-L expression and activity. The editing activity of ADAR1-L on an RNA substrate was not inhibited by LCMV replication. Accordingly, an adenosine-to-guanosine (A-to-G) and uracil-to-cytidine (U-to-C) hypermutation pattern was found in the LCMV genomic RNA in infected cell lines and in mice. In addition, two hypermutated clones with a high level of A-to-G or U-to-C mutations within a short stretch of the viral genome were isolated. Analysis of the functionality of viral glycoprotein revealed that A-to-G- and U-to-C-mutated LCMV genomes coded for nonfunctional glycoprotein at a surprisingly high frequency. Approximately half the GP clones with an amino acid mutation lacked functionality. These results suggest that ADAR1-L-induced mutations in the viral RNA lead to a loss of viral protein function and reduced viral infectivity. This study therefore provides strong support for the contribution of ADAR1-L to the innate antiviral immune response.

Early during viral infections, innate immunity is essential to restrict viral replication and systemic spreading until the virus-specific adaptive immune response can effectively eliminate the virus. One key event in the innate immune responses is the induction of type I and type II interferon (IFN- α/β and IFN- γ , respectively) production by virus-infected cells. IFN- α and IFN- γ bind to their receptors and thereby induce an auto- and paracrine stimulation of antiviral enzymes and proteins, such as the RNA-dependent protein kinase (PKR), 2'-5A-dependent RNase (RNase L), and 2'-5' oligoadenylate synthetase (OAS) (17, 31). In addition, a large isoform of the RNA-specific adenosine deaminase (ADAR1-L) is induced by IFN- α and IFN- γ (12, 18, 27, 38). ADAR1-L deaminates adenosine (A) to inosine (I) in double-stranded RNA (dsRNA) present in the cytoplasm of cells (27, 40). For other isoforms of ADAR, such as the nuclear ADAR1-S that is constitutively expressed, and ADAR2, deamination was found to be highly specific for selected mRNAs within the cell. ADAR1-L function has not been studied in detail in the cell. However, in vitro, ADARs not only exhibit site-specific deamination, but also longer, perfectly matched double-stranded RNA stretches are edited extensively and nonspecifically (28).

A-to-I deamination represents a form of genetic recoding. Inosine is recognized as guanosine by decoding ribosomes and by transcribing polymerases, leading to A-to-G transitions in the edited strand and U-to-C transitions in any cRNA (29). This suggests that ADAR1-L may be an effector molecule of the innate immune response against RNA viruses that repli-

cate via a dsRNA intermediate. ADAR1-L is thought to exert its antiviral activity by introducing errors into the viral genome. In accordance with this hypothesis, an A-to-G/U-to-C hypermutation pattern has been observed in several negative-strand RNA viruses (9, 25, 30, 41). In addition, since inosine prefers to pair with cytidine (C) instead of uracil (U), deamination creates an unfavorable I=U base pair, which changes the structure and stability of deaminated dsRNA. Finally, an RNase that specifically degrades hyperedited inosine-containing RNA has been described and was proposed to be part of a putative antiviral pathway (32, 33).

In this study, we analyzed the genome of the lymphocytic choriomeningitis virus (LCMV), an arenavirus, for mutations indicative of the antiviral activity of ADAR1-L in vivo. Arenaviruses contain two single-stranded genomic RNAs, called S and L, which exhibit an ambisense coding strategy. The short (S; 3.4 kb) segment of LCMV carries the open reading frames for the nucleoprotein (NP; ca. 63 kDa) and viral glycoprotein (GP) precursor (GP-C; 75 kDa). Posttranslational processing of GP-C by the cellular protease S1P generates GP-1 (40 to 46 kDa) and GP-2 (35 kDa) polypeptides (4), which remain non-covalently associated and mediate binding of LCMV to its receptor and fusion with the plasma membrane. The large (L; 7.2 kb) segment codes for the viral RNA-dependent RNA polymerase (RdRp) gene L (ca. 200 kDa) and for the small zinc finger protein Z (11 kDa). Each genome segment contains an intergenic loop, which is predicted to fold into single- or double-stem-loop structures (11) and to terminate transcription of the two genes. LCMV replicates exclusively in the cytoplasm. The RdRp directs synthesis of mostly encapsidated, uncapped full-length antigenomic and genomic RNA species. These encapsidated RNA species serve as templates for the transcriptase activity of the viral RdRp to direct synthesis of subgenomic size, unencapsidated, capped, and nonpolyadenyl-

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ated mRNA for translation of viral proteins (24). During the replication process, an intermediate containing dsRNA is formed, which could be a possible target for ADAR1-L. Infections of mice and cells in culture with LCMV lead to IFN- α induction (6) and hence activate the innate immune response and potentially also ADAR1-L.

In accordance with this hypothesis, PKR and ADAR1 were found to be upregulated after LCMV infection and an A-to-G hypermutation pattern was seen in the LCMV genome in infected cell lines and in acutely infected mice. A substantial fraction of the GP mutants demonstrated partial or full loss of functionality mainly due to A-to-G-induced mutations.

MATERIALS AND METHODS

Mice, cells, and virus. C57BL/6 mice were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany) and were kept under specific-pathogen-free conditions. Animals were cared for in accordance with institutional guidelines and with the German federal law (Tierschutzgesetz). LCMV strain WE (HPI sequence) (3) was used in all experiments. Mice were infected intravenously (i.v.) with 10^5 infectious units (IU), and cell lines were infected with the indicated multiplicities of infection (MOI).

Cell lines, cell culture, and transfection. The cell lines 293T (no. CRL-11268), 293 (no. CRL-1573), TE671 (no. HTB-139), SH-SY5Y (no. CRL-2266), and L929 (no. CCL-1) were obtained from the American Type Culture Collection (Wesel, Germany). These cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine and 10% fetal bovine serum. For transfection, L929 and SH-SY5Y cells were seeded with a density of 1×10^5 cells per well in a 24-well plate. The cells were transfected 1 day later with Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) as described in the product manual. For stimulation with IFN- α , the cells were treated once with 1,000 U/ml human IFN- α (Roche, Mannheim, Germany) or murine IFN- α (Biozol, Eching, Germany).

Production and titration of retroviral pseudotypes. Pseudotyped retroviral vectors were generated by cotransfection of 293T cells with plasmids encoding LCMV GP, murine leukemia virus gag/pol, and a retroviral vector encoding the enhanced green fluorescent protein (EGFP), as previously described (5). Supernatants of the packaging cells were harvested 36 h and 48 h after transfection, and vector titers were measured by end point dilution on TE671 cells, as described previously (5).

Flow cytometry. For staining of LCMV GP on the cell surface, transfected cells were resuspended in phosphate-buffered saline (PBS) 48 h after transfection and incubated for 30 min at 4°C with monoclonal anti-LCMV GP-1-directed antibody KL25 (8). The cells were washed three times with PBS containing 2% fetal calf serum and labeled for 30 min at 4°C with goat anti-mouse antibody conjugated to R-phycoerythrin (Caltag, Hamburg, Germany). Stained cells were subsequently analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany).

Immunoblot analysis. Cells were lysed in lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 2% aprotinin [Sigma, Munich, Germany], 2 mM EDTA [pH 8.0], 50 mM NaF, 10 mM $\text{Na}_4\text{P}_2\text{O}_7 \times 10 \text{ H}_2\text{O}$, 10% glycerol, 1 mM NaVO_4 , 2 mM Pefabloc SC) for 20 min at 4°C and centrifuged for 10 min at 13,000 rpm. The supernatant was mixed at a ratio of 1:2 in 2 \times protein loading buffer (1 \times loading buffer contains 100 mM Tris [pH 6.8], 10% glycerol, 3% sodium dodecyl sulfate, 0.05% bromophenol blue, and 2% β -mercaptoethanol) and heated for 5 min at 95°C. The protein samples were separated by sodium dodecyl sulfate-7% or 10% polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with PBS containing 5% dried nonfat milk powder and incubated with the monoclonal rat anti-hemagglutinin (anti-HA) tag-specific antibody 3F10 (Roche, Mannheim, Germany), polyclonal rabbit anti-ADAR1 antibody (Zymed), or rabbit anti-phospholipase C γ (PLC- γ) antibody (BD Pharmingen, Heidelberg, Germany). After washing of the membranes with PBS and incubation with horseradish peroxidase-conjugated goat anti-rat antibody or goat anti-rabbit antibody (Dianova, Hamburg, Germany), the proteins were detected by chemiluminescence with the ECL enhanced chemiluminescence system (Amersham-Pharmacia, Freiburg, Germany).

RT-PCR. RNA was isolated from cell lines or murine spleen tissue with the RNeasy mini kit (QIAGEN, Hilden, Germany) according to product description. A DNase digestion was performed during the RNA isolation with DNase I

(Invitrogen, Karlsruhe, Germany). Reverse transcription (RT) to generate cDNA was performed with Superscript II (Invitrogen, Karlsruhe, Germany) and random primers (Invitrogen, Karlsruhe, Germany) as described in the product manual. The cDNA was then used for amplification with PFX polymerase (Invitrogen, Karlsruhe, Germany) or for quantitative PCR (qPCR) with the iQ SYBR green supermix (Bio-Rad, Munich, Germany). The primers Piup (5'-GATATCGCGGGCGCAATGAATCC-3') and ADAR1-Low/sbf1 (5'-CCTGCAGGAGCATACTATACTGGGAGAGATA-3') were used to amplify the full-length open reading frame of ADAR1-L from HeLa cell cDNA. For cloning of the GluR-B expression vector pMPB13, the primers B13for (5'-CCTAGGAGATCTGGATGTGCATTG-3') and B13rev (5'-CCTAGGTCTAGATATGTGTGATC-3') were used. For amplification of clones of the LCMV S-RNA, the primer S-RNA up29 (5'-TGCGCTTCTCTTATAGGACAAGTGG-3') and either S-RNA low861 (5'-GGTGAATGTGCCTGAAAGTCTCT-3') or S-RNA low4 (5'-ATGAACCGCCCTTATGTGTC-3') were used in a 30-cycle amplification protocol: 94°C for 180 s, 30 times (94°C for 30 s, 58°C for 30 s, and 68°C for 60 s or 120 s), and 68°C for 240 s. The PCR product was cloned for sequencing into pCR-Blunt II-TOPO vector (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. With this protocol, either full-length LCMV GP clones or clones coding only for LCMV GP-1 were generated. The qPCR specific for ADAR1-L was performed with mADAR up1 (5'-AGCCCCCTGGAGTTGTGAATC-3') and mADAR low1 (5'-GGAGTTGCCCTTGCCTGTAGTC-3'); for murine PKR with mPKR up1 (5'-ATTGGCTTAGGTGGATTGGTCA-3') and mPKR low1 (5'-GTGCTCCGCCCTTCTCCGTGTTAT-3'), and for viral S-RNA with S-RNA up5 (5'-CAAGGTGCCTGGTGTAAAACTAT-3') and S-RNA low4. The samples were normalized against mGapDH using the primers mGapDH up (5'-CATGGCCCTTCGGTGTTCCTA-3') and mGapDH low (5'-GCGGCACGTCAGATCCA). The reaction was performed on an iCycler (Bio-Rad, Munich, Germany) with a two-step amplification protocol after an activation step for 180 s at 94°C followed by 50 cycles of 94°C for 30 s and 58°C for 45 s and analyzed with qgene96 macro.

Construction of the ADAR1-L, S-RNA, GluR-B, and LCMV-GP expression plasmids. The PCR product of ADAR1-L amplified from HeLa cell cDNA with the primers Piup and ADAR1-Low/sbf1 was cloned in a pCR2.1-TOPO vector with the TOPO-TA cloning kit (Invitrogen, Karlsruhe, Germany) and subcloned into pcDNA3.1 with NotI and SpeI to yield pAD1. The S-RNA expression plasmid was constructed by cloning of the S-RNA from the plasmid PY1A into the vector pHH21 (26) to yield the plasmid M236. The GluR-B plasmid was constructed from the B13 plasmid (kindly provided by M. Higuchi) (15) by amplification of a part of the GluR-B coding sequence with the B13for/rev primers. This PCR product was cloned with AvrII into the expression vector MP1-eGFP (34) to obtain the pMPB13 plasmid. The control plasmid pcMPB13 was obtained by amplification of cDNA isolated from pMPB13-transfected cells with B13for/rev and cloning of the PCR product into pCR-Blunt II-TOPO. The LCMV GP expression plasmids were cloned out of the pCR-Blunt II-TOPO sequencing vector either into the expression vector pEF α under the control of an EF1- α promoter or the plasmid pCMV under the control of the CMV promoter, which in addition contains a β -globin intron.

ADAR1-L editing activity assay. SH-SY5Y cells were transfected with 1 μg of the plasmids pMPB13 and pAD1. One day after transfection, the cells were infected with LCMV. Two days p.i., RNA was extracted with the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the product description. cDNA was generated with Superscript II, and a PCR with *Taq* polymerase and the B13for/rev primers was performed. The PCR products were purified with the E.Z.N.A. Cycle-Pure kit (Peqlab, Erlangen, Germany) according to the product description and sequenced with the B13for primer on a 3100 Avant genetic analyzer (Applied Biosystems, Darmstadt, Germany). The amount of editing activity was calculated from the ratio of guanosine to adenosine peak at the +60 site of GluR-B. Different mixtures of the plasmids pMPB13 and pcMPB13 were amplified in the same PCR to construct a calibration curve from 0 to 100% editing at the +60 site of GluR-B.

ELISA. At the indicated days after infection, mice were bled and serum was prepared to determine the level of IFN- α by enzyme-linked immunosorbent assay (ELISA; PBL Biomedical, Piscataway NJ) according to the product description.

RESULTS

Predominant A-to-G and U-to-C mutations in LCMV-infected cells and mice. Viruses such as polyomavirus (20), measles virus (41), human parainfluenza virus 3 (25), and respiratory syncytial virus (30) show an A-to-G hypermutation pattern

in their genome mostly in the late state of infection. These observations have never been proven to be a direct result of ADAR1-L activity. Here, the mutation rate and pattern of genomic clones of LCMV early and late after infection of L929 cells were analyzed. We focused on the GP sequence to extend the number of sequenced clones and to determine functional consequences of the mutations detected. Murine L929 cells were infected with LCMV at an MOI of 0.1, and the sequences of 70 LCMV RNA clones were determined. The sequencing of viral RNA clones was necessary because the direct sequencing of the PCR product did not reveal any specific mutation, which indicates that no genetic shift or mutational hot spots appeared within the viral genome. Two days after infection, the mutations did not exhibit any specific pattern (Fig. 1A). Seven days p.i., a distinct A-to-G mutation bias emerged. Furthermore, two of the 54 analyzed clones showed an A-to-G or U-to-C hypermutation pattern in a very short stretch of the GP where 38% of all adenosines or 14% of uracils were mutated (see Fig. S1 in the supplemental material). Since we did not distinguish between genomic and antigenomic sequences of LCMV during cDNA synthesis, A-to-G mutations in the antigenome are seen as U-to-C mutations in the genome of LCMV and vice versa. We therefore scored these mutations as equivalent. To minimize polymerase errors during the PCR, we used PFX polymerase with a 3'-to-5' proofreading activity for amplification of cDNA, since *Taq* polymerases are known to generate preferentially A-to-G transitions (7). In addition, we performed a control RT-PCR with RNA expressed in L929 cells from a plasmid encoding for the S-RNA of LCMV. Here, we did not find a specific mutation pattern in the analyzed clones but a random distribution with more transitions than transversions (Fig. 1A). This is a general bias, which was detected with several DNA polymerases (1, 19). Therefore, we can exclude induction of specific mutations by the reverse transcriptase and polymerase.

To validate these cell culture data *in vivo*, we infected C57BL/6 mice with LCMV and analyzed 213 viral genomic clones for mutations. The clones were isolated from spleen tissue, a site of high viral replication. Four days p.i., a clear preference for A-to-G/U-to-C transitions was detected (Fig. 1B), whereas no G-to-A/C-to-U transition occurred. This A-to-G/U-to-C mutation preference was still pronounced on day 5 and day 8 p.i., though the rate of G-to-A/C-to-U transitions also increased. The higher frequency of G-to-A mutations at late time points of infection may reflect a generally higher level of transitions (92%) versus transversions (8%). This mutation pattern is most likely the result of misincorporation of nucleotides by the viral polymerase. In LCMV clones isolated from mice, we did not find a hypermutated LCMV clone. This may be due to a faster degradation of such clones, which are known to be targeted by nucleases that cleave inosine-containing RNA (33). The overall mutation rate of the viral genome in mice was generally lower than that in L929 cells (Table 1), possibly reflecting the higher selection pressure for functional virions *in vivo*. In both systems, A-to-G/U-to-C transitions were the major mutation type in the GP sequence of LCMV. Half of the LCMV GP clones derived from L929 cells contained at least one amino acid (aa) mutation, whereas of the clones derived from organs of mice, only ~20% had 1 or more aa mutations.

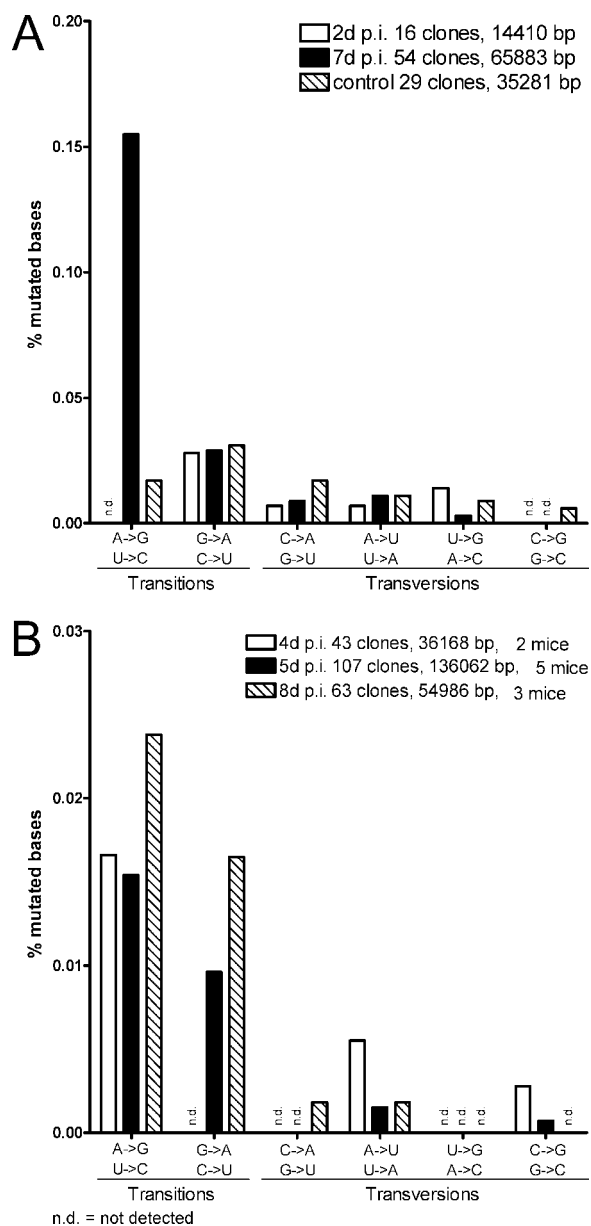


FIG. 1. A-to-G/U-to-C mutations dominate in LCMV RNA of infected L929 cells and mice. (A) L929 cells were infected in a 24-well plate with LCMV WE at an MOI of 0.01. Two and 7 days p.i., cells were lysed and RNA was extracted. For a control, L929 cells transfected with the plasmid M236 encoding the S-RNA of LCMV under the control of the polI promoter were used. A PCR with a proofreading polymerase was performed with two different primer sets, and the product was cloned into a sequencing vector. The bars represent the percentage of all mutations found in two (2 days) and three (7 days) separate experiments. The total numbers of clones and base pairs sequenced are given. (B) C57BL/6 mice were infected i.v. with 1×10^5 infectious units of LCMV WE. The mice were sacrificed at the indicated time points, RNA was isolated from spleen, and a PCR was performed as described for panel A. The bars represent the percentage of mutations of the LCMV GP clones. The numbers of mice, clones, and base pairs analyzed are given. The data were compiled from two independent experiments.

ADAR1-L is up-regulated in L929 cells and mice after infection with LCMV. The IFN- α -mediated antiviral response can be inhibited by viral proteins or RNAs from a broad range of viruses (17). Though such inhibitory molecules are not

TABLE 1. Error rate and overview of mutated clones

Group	Error rate (10 ⁻⁴)	% A-to-G/U-to-C transitions	No. of clones with aa mutations ^a
L929 cells (7 days p.i.)	20	75	27 (13) of 54
C57BL/6 mice			
4 days p.i.	2.49	67	8 (6) of 43
5 days p.i.	2.28	57	20 (11) of 107
8 days p.i.	4.39	54	14 (9) of 63

^a Clones in parentheses have at least 1 aa mutation due to A-to-G (U-to-C) transition.

known for LCMV, we analyzed the induction of ADAR1-L and PKR in response to LCMV infection by quantitative PCR. In L929 cells, ADAR1-L and PKR are upregulated already 24 h after infection with LCMV (Fig. 2A). Forty-eight hours

p.i., expression peaks are fourfold above baseline for ADAR1-L and twofold above the baseline for PKR and then they drop to basal levels at 72 h p.i.. Stimulation with 1,000 U IFN- α leads to a more pronounced (8- to 10-fold) and prolonged induction over a period of 72 h. Expression of ADAR1-L is also upregulated in the spleens of C57BL/6 mice after LCMV infection (Fig. 2B). ADAR1-L expression peaks 48 h p.i. with a 3.5-fold induction and then declines to baseline levels at day 5. PKR shows a similar activation kinetic but is induced to higher levels (12-fold), peaking between days 1 and 3 p.i. and reaching baseline levels again at day 7. Expression levels of PKR and ADAR1-L correlate with IFN- α levels in the serum of the mice, which peak 2 days p.i. (Fig. 2C). IFN- α serum levels then drop to baseline until day 7 p.i., in concordance with the role of IFN- α as an early mediator of innate

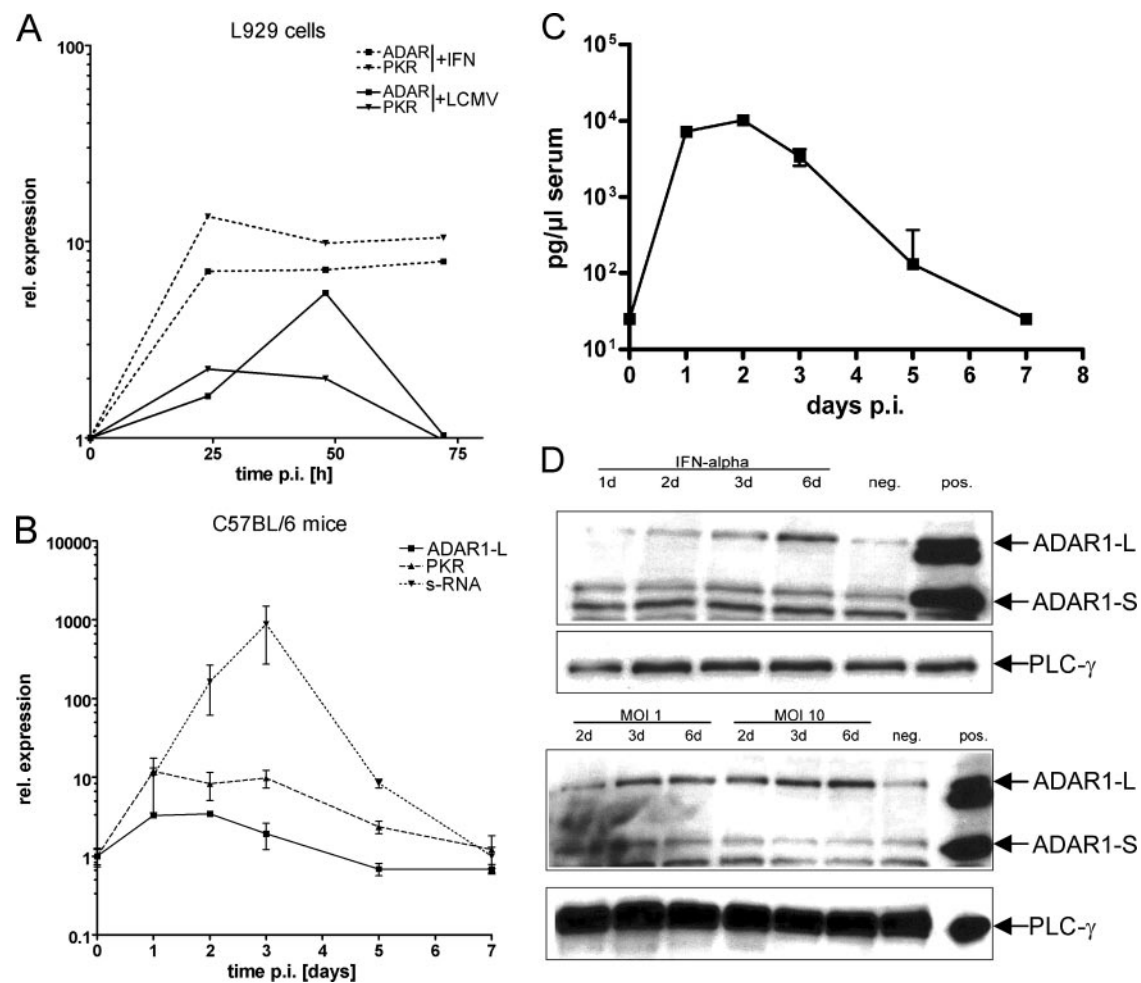


FIG. 2. LCMV induces ADAR1-L and IFN- α expression. (A) L929 cells were infected with LCMV at an MOI of 0.1 or stimulated with 1,000 U murine IFN- α . At the indicated time points, RNA was isolated for subsequent reverse transcription reaction and qPCR with specific primers for ADAR1-L and PKR of LCMV. (B) C57BL/6 mice were infected with 1×10^5 IU LCMV WE i.v. At the indicated time points, three to five mice were sacrificed and RNA was isolated from homogenized spleens. A reverse transcription reaction followed by qPCR specific for ADAR1-L, PKR, and the S-RNA of LCMV was performed. The error bars are the standard error of the mean from three to five animals. (C) C57BL/6 mice were infected with 1×10^5 IU LCMV WE i.v. At the indicated time points, blood from three to five mice was drawn and serum was analyzed for murine IFN- α by ELISA with a detection limit of 25 pg/ μ l. The error bars represent the standard error of the mean from three to five animals. (D) 293T cells were infected with LCMV (MOI 1 and MOI 10) or stimulated with 1,000 U human IFN- α . At the indicated time points, cells were harvested and a Western blot was performed with an antibody specific for ADAR1. The Western blot was reprobed after stripping with an antibody against PLC- γ as a loading control. The positive control is 293T cells transfected with the plasmid pAD1, encoding ADAR1-L expressed under the control of a CMV promoter.

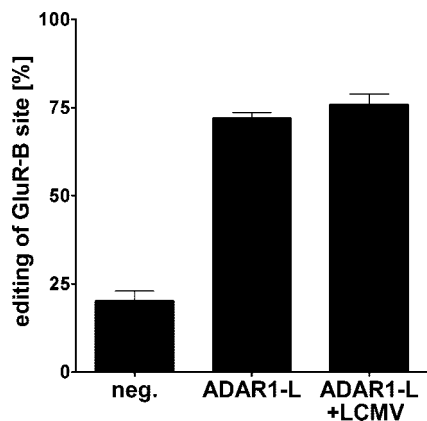


FIG. 3. LCMV does not inhibit ADAR1 activity. SH-SY5Y cells were transfected with the plasmid pMPB13 coding for a part of the GluR-B receptor containing the +60 editing site and a plasmid coding for ADAR1-L. The cells were infected with LCMV (MOI 0.1) and lysed 2 days p.i. RNA was isolated, and an RT-PCR was performed with the B13for/rev primers. The PCR product was sequenced, and the ratio of the guanosine to adenosine peak at the +60 site was determined. For control of the PCR, different mixtures of the plasmids pMPB13 and control pcMPB13, which contains a GluR-B already edited at the +60 site, were also amplified. These PCR products were also sequenced, and a calibration curve (reaching from 0 to 100% guanosine at the +60 site) was created from the ratio between A and G peaks to calculate the amount of editing in the samples.

immune response. To monitor the infection status of the mice, we used a quantitative PCR specific for viral S-RNA. This assay has a higher sensitivity than the plaque assay but it does not discriminate between replication-competent and -incompetent viral RNA. Maximum viral S-RNA expression was reached 3 days p.i. and declined simultaneously with elimination of the virus and IFN- α levels in the serum.

To demonstrate that the induction of ADAR1-L RNA expression by LCMV infection also leads to increased ADAR1-L protein levels, we performed a Western blot analysis. LCMV-infected human 293 cells were used, as there was no antibody for murine ADAR1-L available. As a control, ADAR1-L protein levels after stimulation with IFN- α were analyzed. As expected, ADAR1 protein expression was found to be elevated after IFN stimulation for a period of 6 days (Fig. 2D). 293 cells infected with LCMV at MOIs of 1 and 10 also expressed higher ADAR1-L protein levels than control cells. ADAR1-L expression increased over a period of 6 days p.i.. Accordingly, the maximum rate of A-to-G/U-to-C mutations was found 2 to 5 days after the peak of mRNA expression in mice and in L929 cells, when high levels of ADAR1 protein had accumulated, as shown here for 293 cells. The smaller form of the ADAR1, ADAR1-S (110 kDa), which is constitutively expressed, was not induced by IFN- α or by infection of 293 cells with LCMV.

As some viruses are found to express RNA or protein that inhibits ADAR1 activity, we wanted to rule out inhibition of ADAR1-L editing activity by LCM viral factors (21, 22). We measured editing activity of ADAR1 with plasmid-expressed RNA encoding a part of the B subunit of the glutamate receptor (GluR-B) containing the intron hot spot +60. This +60 site is specifically edited up to 87% in the brain and in vitro by ADAR1 (15, 23). Compared to endogenous cellular RNA tar-

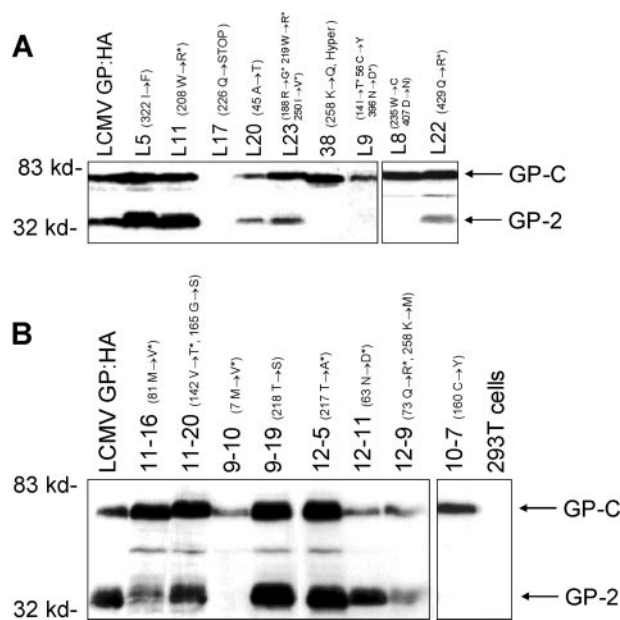


FIG. 4. Mutated LCMV GP clones show reduced processing of GP-C in GP-1 and GP-2. Eighteen mutated GP clones from infected L929 cells (7 days p.i.) or mouse spleens (8 days p.i.) were randomly picked and cloned from the sequencing vector into the expression vector pEF α or pCMV. For detection purposes, an HA tag was C-terminally fused to LCMV-GP. One microgram of each plasmid was transfected into 293T cells, and 48 h after transfection, cells were harvested and equal cell numbers per sample were used for a Western blot analysis probed with an anti-HA antibody. Panel A shows nine LCMV GP clones isolated out of LCMV-infected L929 cells, and panel B shows eight LCMV GP clones isolated from infected mice. As a control (LCMV GP:HA), cells transfected with the wild-type LCMV GP expression vector were used. Asterisks mark amino acid mutations due to A-to-G or U-to-C transitions.

gets for ADAR1-L, this ectopic overexpressed RNA better reflects the ability of LCMV to inhibit editing of a high number of RNA target molecules. We transfected neuroblastoma SH-SY5Y cells with plasmids encoding for a part of GluR-B and ADAR1-L. The cells expressing ADAR1-L showed a fourfold increase in editing activity at the +60 site compared to control cells (Fig. 3). Infection of ADAR1-L-expressing SH-SY5Y cells with LCMV (MOI of 0.1) did not alter the amount of editing at the GluR-B +60 site. Thus, LCMV was not found to interfere with the editing activity of ADAR1-L.

Mutations in the viral glycoprotein lead to nonfunctional protein. Up to 50% of the mutations found in the RNA coding for LCMV GP in L929 cells and mice lead to mutations on the protein level (Table 1). To determine the functionality of these LCMV glycoprotein mutants, the clones were transferred into an expression vector and then analyzed for processing, surface expression, and pseudotyping capacity for retroviral vectors. Thereby, the impact of genomic mutations on the functionality of viral GP and especially the potential contribution of ADAR1-L editing to the loss of GP function could be assessed. Processing of the viral precursor glycoprotein GP-C into GP-1 and GP-2 is essential for surface expression and pseudotyping capacity (3). Five of 17 mutated clones analyzed in total were not cleaved by the cellular subtilase SKI-1/S1P or were not detectable by Western blotting (Fig. 4). An additional four

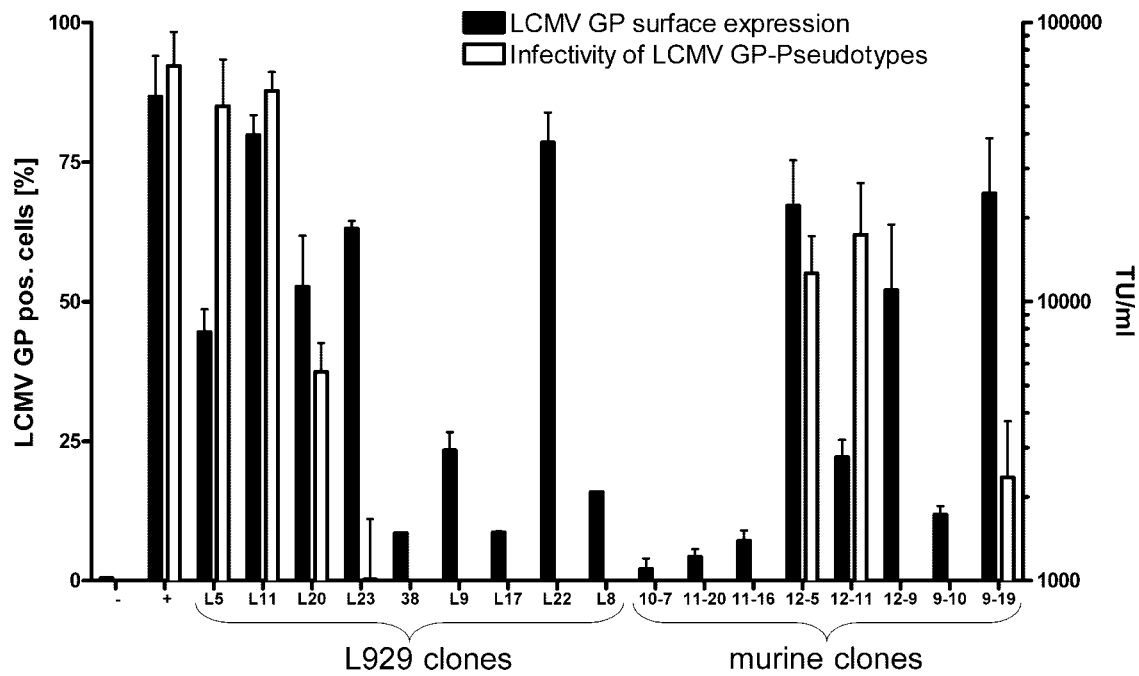


FIG. 5. Surface LCMV GP expression and pseudotyping capacity of the mutated LCMV GP clones. 293T cells were transfected with a plasmid coding for the mutated LCMV GP, a retroviral vector containing eGFP as marker gene, and a plasmid for the retroviral gag/pol. Supernatants of the transfected cells were collected 36 h and 48 h after transfection and pooled. The black bars represent the LCMV GP surface expression 48 h after transfection detected by GP-specific monoclonal antibody in flow cytometry analysis. The white bars represent the eGFP transducing units (TU) of the collected supernatants titrated on TE671 cells with a lower detection limit of ~1,000 TU/ml. The graph represents the mean (\pm standard deviation) of two experiments, each done in duplicate. The negative control is 293T cells transfected without a plasmid coding for LCMV GP, and the positive control is cells transfected with the wild-type LCMV GP plasmid.

clones were cleaved in a reduced fashion with a weaker GP-2 band compared to GP-C than the control. Furthermore, four clones showed altered migration of GP-C suggestive of an altered glycosylation pattern. All LCMV GP clones that were not cleaved into GP-1 and GP-2 also had reduced or absent surface expression of the LCMV GP on the cell surface and were not able to pseudotype a retroviral vector (Fig. 5). In contrast, 10 of the 12 clones that were processed correctly were expressed on the cell membrane at a level comparable to that of the control. However, 4 of these 10 clones were not able to pseudotype the retroviral vector efficiently. Two processed clones showed reduced GP cell surface expression but were able to pseudotype retroviral vectors. One of the A-to-G hypermutated clones (clone 38) was also analyzed and, as expected, had neither surface expression nor pseudotyping capacity (Fig. 6). Thus, massive hypermutation, even if the open reading frame is preserved, abolishes viral protein function.

Ten out of the 17 analyzed clones contained one or more amino acid mutations caused by A-to-G or U-to-C transitions at the RNA level. Only three of these coded for fully functional glycoprotein. A-to-G mutations were responsible for 58% of the nonfunctional LCMV GP clones in L929 cells and 63% of them in mice. We conclude that A-to-G/U-to-C induced mutations can impair viral protein function and weaken viral fitness. Thereby, ADAR1-L may contribute to the innate defense against viruses. However, it must be noted that 69% of the GP clones produced in infected L929 cells and 91% of the clones isolated from infected mice were functional (Table 2). Thus, even if one assumes that the functionality of the other three

viral proteins (NP, RdRp, and Z) is similarly sensitive to mutations as that of the glycoprotein, the introduction of errors into the coding viral sequences alone cannot in itself control virus infections. This mode of action must be seen as one

clone	mutated amino acids			GP-C processing	LCMV GP surface expression	pseudotyping capacity
L9	14 I→T*	56 C→Y	398 N→D*	n. p.	(+)	-
L20	45 A→T			+	(+)	(+)
L11	208 W→R*			+	+	(+)
L23	186 R→G*	219 W→R*	250 I→V*	(+)	(+)	(+)
L17	226 Q→STOP			-	-	-
L8	235 W→C	407 D→N		n. p.	-	-
38	258 K→Q	Hypermutation*		n. p.	-	-
L5	322 I→F			+	+	+
L22	429 Q→R*			(+)	(+)	-
9-10	7 M→V*			n. p.	(+)	-
12-11	63 N→D*			(+)	(+)	+
12-9	73 Q→R*	258 K→M		+	+	-
11-16	81 M→V*			(+)	-	(+)
11-20	142 V→T*	165 G→S		(+)	-	-
10-7	160 C→Y			n. p.	-	-
12-5	217 T→A*			+	+	+
9-19	218 T→S			+	+	(+)

FIG. 6. Summary of the analyzed LCMV GP clones. The different amino acid mutations in LCMV GP of each analyzed clone are shown. Asterisks mark amino acid mutations due to A-to-G or U-to-C transitions. Processing of the mutated GP-C into GP-1 and GP-2 equal to that of the nonmutated LCMV GP is marked +, and moderate processing is marked (+). Nonprocessed GP-C is indicated by n.p. Mutated LCMV GP surface expression and pseudotyping capacity are also marked with + if equal to those of nonmutated LCMV GP, with (+) if lower than those of the control, and with - if not detectable.

TABLE 2. Frequencies of defective LCMV GP

Group (day p.i.)	% of LCMV GP					
	Infectious mutants				Noninfectious mutants	
	Not mutated	A-to-G/ U-to-C mutated	Other mutations	Total	A-to-G/ U-to-C mutated	Other mutations
L929 cells (7)	50	6	13	69	18	13
C57BL/6 mice (8)	82	6	3	91	6	3

contribution within the complex network of IFN-induced antiviral effector functions.

DISCUSSION

The primary finding of this study was the emergence of a distinct A-to-G/U-to-C hypermutation pattern in the single-stranded RNA genome of LCMV both in cell culture and in the organs of infected mice. This pattern is probably due to unspecific deamination by ADAR1-L, which was found to be upregulated during infection of cells and mice with LCMV. Furthermore, these A-to-G mutations lead to a high percentage of nonfunctional viral protein, thereby weakening viral fitness.

The A-to-G hypermutation pattern observed in this study was most likely attributed to RNA editing and not to errors of the viral polymerase. The hypermutation pattern was not observed 2 days after infection in L929 cells. In mice at days 5 and 8 p.i., the dominance of A-to-G mutations is also reduced compared to that at day 4, which is consistent with lower ADAR1-L expression at these time points. Furthermore, although viral RNA polymerases lack 3'-to-5' proofreading activity, they have never been reported to exhibit a specific mutation pattern (35, 37). To further rule out methodological errors induced during amplification of viral clones, the PFX polymerase with proofreading activity was used instead of *Taq* polymerase, which is prone to induce frequent A-to-G mutations (7). The most important finding in favor of ADAR-dependent effects in LCMV-infected cells was that the sequence of LCMV GP cDNA derived from RNA transcribed from a classical expression plasmid was not mutationally biased towards A-to-G mutations. Only a preference for transitions was detected, which is typical for many polymerases (1, 19). In support of ADAR1-L editing of the viral genome, we could not detect an A-to-G/U-to-C hypermutation pattern in ADAR1 null mouse embryonal fibroblast cells of C57BL/6 mice. However, the corresponding wild-type mouse embryonal fibroblast expressed extremely low levels of ADAR1 and did not show a clear mutational bias (data not shown). We therefore cannot completely exclude that the lack of mutational bias in the ADAR1 null cells is caused by another factor specific for embryonal fibroblasts of this mouse strain. This issue requires further investigation. Taken together, the emergence of hypermutated LCMV GP clones isolated from infected murine L929 and human 293T cells (see Fig. S1 in the supplemental material) is highly suggestive for unspecific editing of the viral RNA by ADAR1-L. A general unspecific deamination of isolated dsRNA longer than 50 bp by ADAR was already demonstrated in cell-free systems (28). Domingo and colleagues have also

found an increased occurrence of A-to-G/U-to-C mutations in the genome of LCMV (14), and a recent study revealed one LCMV clone with a high degree of U-to-C mutations (13) in the viral polymerase gene. Furthermore, a similar mutation pattern was detected in several RNA (e.g., measles virus and respiratory syncytial virus) (9, 25, 30, 36, 41) and DNA (e.g., polyomavirus) (20) viruses, which was postulated to result from ADAR1 editing.

The numbers of A-to-G mutations are probably underestimated in our study. Inosine-rich RNA produced by extensive ADAR1-L activity is a target for specific cleavage by cellular nucleases (33). Thus, it is not surprising that hypermutated LCMV GP clones are not detected at a higher frequency than the 3.7% seen in L929 cells. Furthermore, binding of the primers used to amplify the viral S-RNA will be inefficient for hypermutated RNA sequences. In viral RNA isolated from LCMV-infected mice, no hypermutated clones were found. Since ADAR1-L is induced in murine spleen by infection with LCMV to a similar extent as in L929 cells, a simple lack of editing activity is most likely not the reason. However, the low rate of detected mutations in these clones (though A-to-G mutations are the most frequent mutation type) is indicative of a higher selection pressure in vivo for functional viral RNA. Possibly the effective concentration of infectious virus in animals is lower than that in cell culture and defective genomic RNAs cannot propagate due to a lack of helper virus function. In addition, those virus particles that do not efficiently and rapidly bind to host cells might be eliminated by macrophages. Furthermore, activity of inosine-specific nucleases could be higher in vivo, resulting in increased degradation of deaminated RNA.

ADAR1-L editing has been postulated to have several functions in innate immunity. First, A-to-G hypermutation of the coding viral genome may lead to the production of mutated, functionally impaired viral proteins. In addition, deamination generates base mismatches, which can disturb functionally important secondary structures of the viral genome. Finally, edited viral RNA is effectively degraded by cellular nucleases that target inosine-rich RNA (32, 33). In this study, we show that indeed a high percentage of the GP mutants in infected cells and mice are functionally inactive. Furthermore, if one assumes that the other regions of the viral genome are mutated at frequencies similar to those of the analyzed GP coding sequence the L, S, and Z proteins would also be expected to have reduced functionality. The loss of functional GP was mainly due to insufficient cleavage of the GP-C in GP-1 and GP-2 and a markedly reduced surface expression. This most likely reflects a misfolding of the mutated protein, which then is redirected to degradation in the cytoplasm without reaching the late or post-Golgi compartment, where the subtilase SKI-1/S1P cleaves GP-C (4). In addition, misfolded protein could accumulate and induce an unfolded protein response leading to a shut down of cellular protein synthesis, growth arrest, and, finally, apoptosis (42). The activation of the unfolded protein response has already been described for the paramyxovirus simian virus 5 (39) and bovine viral diarrhea virus (16).

The proposed role of ADAR1-L in directly combating viruses by editing of viral RNA has never been unambiguously confirmed since the first description of U-to-C hypermutated measles virus genomes in 1988 (10). Several studies have shown that viruses have coevolved with the immune system and developed several

countermeasures against antiviral strategies of the cell, including ADAR1 editing (2, 31). This indeed indicated that editing may be part of the innate antiviral machinery. For LCMV, however, we did not find an inhibition of ADAR1 activity by LCMV replication. The first direct proof of suppression of viral replication by ADAR activity has only recently been presented for the HCV replicon (36). However, wild-type viruses have never been shown to lose replication competence by ADAR1-L editing activity. Here, we show that hypermutation can indeed impair viral protein function. This study represents a further advance in our understanding of the contribution of ADAR1-L to the innate immune response against viral infections.

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